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Induction of the Base Displacement or Z Conformation in DNA by *N*-2-Acetylaminofluorene Modification

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Modification of deoxyguanosine at the C_8 position by the carcinogen *N*-acetoxy-*N*-2-acetylaminofluorene (N-AcO-AAF) has been shown to result in two different conformational changes dependent on the nucleotide sequence of the modified polymer. AAF modification of random sequence DNA results in a large distortion of the helix which is termed base displacement. In this conformation, the carcinogen is inserted into the DNA perpendicular to the helix axis with the guanosine displaced to the outside. Large single-stranded regions are generated which are susceptible to S_1 nuclease digestion and react with anti-cytidine antibodies.

A different conformation has been observed when the alternating purine pyrimidine copolymer, poly(dG-dC)·poly(dG-dC) is modified. At a modification level of 28% this polymer shows a CD spectrum characteristic of the left-handed Z-DNA seen in the unmodified polymer at high ethanol or salt concentrations. Base pairing of the modified polymer remains intact as demonstrated by its resistance to digestion with S_1 nuclease and lack of reactivity with anti-cytidine antibodies.

Modification of poly(dG-m^{5}dC)·poly(dG-m^{5}dC) with AAF was also shown to induce the Z conformation. However, for this polymer, inversion of the CD spectrum takes place at a much lower modification level (10%) than for the nonmethylated polymer (>20%). This polymer is also resistant to S_1 nuclease digestion consistent with its adoption of the Z conformation with AAF modification. A possible role in gene expression for the Z conformation of AAF modified regions is discussed.

Introduction

The reaction of the carcinogen *N*-acetoxy-*N*-2-acetylaminofluorene (N-AcO-AAF) at the C_8 position of deoxyguanosine residues in native DNA has been shown to result in large conformational changes in the polymer which is expressed in a base displacement model (*1*). In this model, the attachment of AAF results in a change in glycosidic N^9-$C^{1'}$ bond from the *anti* conformation of nucleosides with Watson-Crick geometry to the *syn* conformation. The AAF residue is inserted into the helix and stacked with the adjacent base while the guanine residue is displaced to the outside of the helix. A similar model called insertion-denaturation has been proposed by others (*2*). The result is a marked distortion of the double-stranded helix at the sites of AAF modification and generation of local regions of denaturation. This has been shown by the increased susceptibility of AAF-modified DNA to digestion by S_1 nuclease, a single strand specific endonuclease (*3*, *4*). A stereoscopic view of the base displacement model is shown in Figure 1.

Recently, a new family of left-handed Z helical structures has been described (*5-7*) (Fig. 2) based on X-ray diffraction studies of alternating d(CpG)-DNA crystals. Differences in the conformation of the alternating deoxyguanosine and deoxycytidine residues results in a dinucleotide repeating unit, not a mononucleotide, as in B-DNA. In Z-DNA, the deoxyguanosine is in the *syn* conformation, while the deoxycytidine is in the *anti* conformation. The deoxyribose ring of cytidine has a pucker in which the 2′ carbon is in the *endo* conformation, while that of guanosine can be C3′ *endo* (*5*) or C1′ *exo* (*7*) depending on whether the sample was crystalized from low or high salt solutions, respectively.

In solution, poly(dG-dC)·poly(dG-dC) undergoes a

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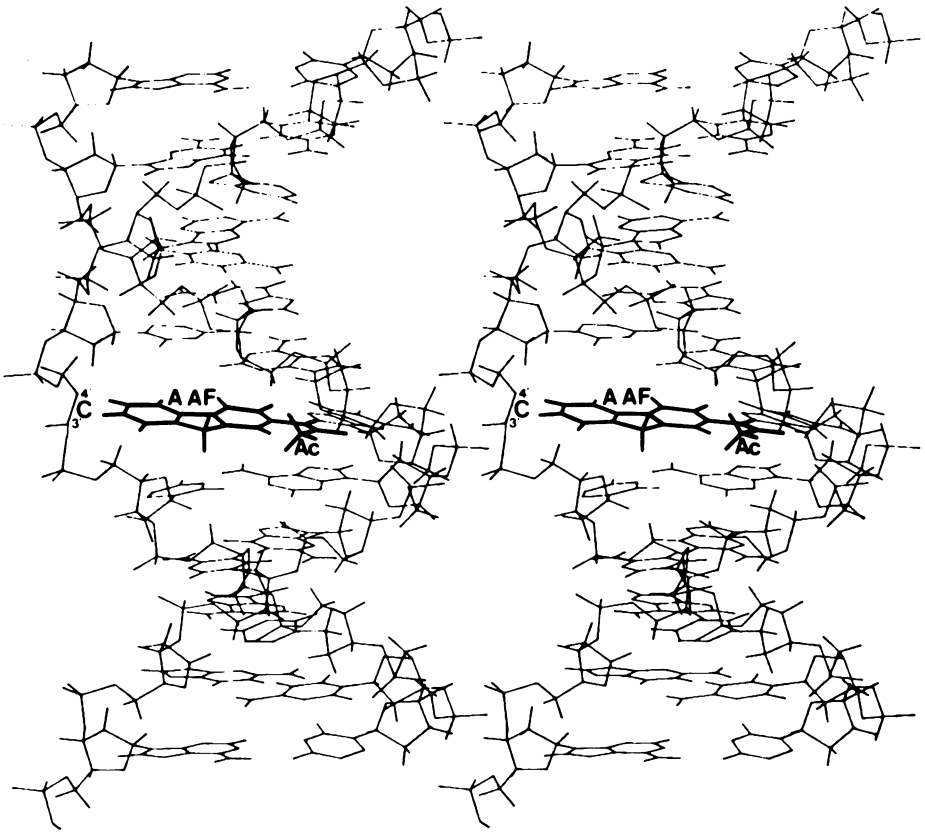

FIGURE 1. Stereoscopic view of the base displacement model of AAF-DNA adducts. The guanine to which the AAF is attached has been rotated out of the helix and the AAF moiety is inserted into the helix and stacked with the bases above and below. AC-designates acetyl group of AAF. The cytosine (marked C) residue on the opposite strand would overlap with the AAF residue; therefore it has been removed and the 3′ and 4′ carbon atoms of the corresponding deoxyribose in the DNA backbone are indicated. In reality, this C probably rotates out from the helix to accommodate the AAF; the exact conformation is not known, although there is evidence that this region of DNA is "single-stranded." To view this image in stereo, two lenses of about 20 cm focal length should be mounted about 14 cm above the images and 6.5 cm apart. Viewers of this type are sold by Taylor Merchant Corp., 24 W. 45 St., New York, NY 10026 under the name Stereoptician 707.

salt-(*8*) or ethanol-induced (*9*) conformational change as measured by circular dichroism (CD). The CD spectrum of the high-salt form is virtually an inversion of that of the low-salt (B-DNA) form. [^{31}P]NMR spectra (*10*) show two peaks in high salts which suggest different phosphodiester linkages and a dinucleotide repeating unit. Several other studies have provided additional evidence that the high salt or ethanol solution structures correspond to the Z-DNA structure of the crystals (*11, 12*).

As a direct consequence of the *syn* conformation of the deoxyguanosine residues in Z-DNA, the C_8 position is exposed on the outer surface of the molecule (Fig. 3). Modification of this position by AAF may, therefore, not require such drastic distortion of the conformation as is seen in B-DNA where the C_8 position is crowded inside the helix. Z-DNA may, therefore, be more susceptible to carcinogen modification than B-DNA. In addition, since AAF modification of deoxyguanosine in DNA results in the rotation of the guanine from *anti* to the *syn* conformation, a similar change in conformation would be expected in poly(dG-dC)·poly(dG-dC) and might force the conformation of the entire polymer into the Z form.

Conformation of AAF-Modified Poly(dG-dC)·poly(dG-dC)

We have investigated the conformational changes induced in poly(dG-dC)·poly(dG-dC) with AAF modification (*13*). Figure 4 shows the CD spectra of poly(dG-dC)·poly(dG-dC) in buffer and 60% ethanol. In aqueous solution, a B-DNA spectrum is seen while in ethanol the CD is inverted and characteristic of Z-DNA. Also shown is the CD spectrum of a sample modified to the extent of 28% with AAF. With this high level of modification, the CD spec-

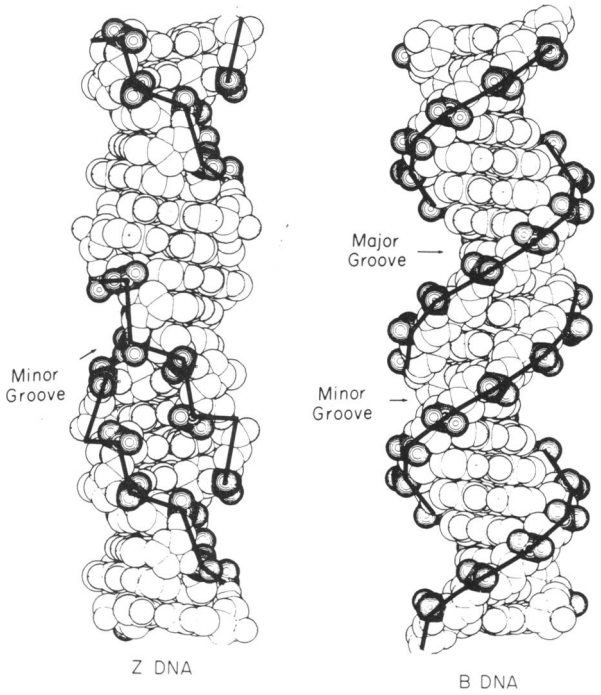

FIGURE 2. van der Waals side views of Z-DNA and B-DNA. The irregularity of the Z-DNA backbone is illustrated by the heavy lines which go from phosphate to phosphate residues along the chain. The minor groove in Z-DNA is quite deep, extending to the axis of the double helix. In contrast, B-DNA has a smooth line connecting the phosphate groups and two grooves, neither one of which extends into the helix axis of the molecule. From Wang et al. (*5*) with permission.

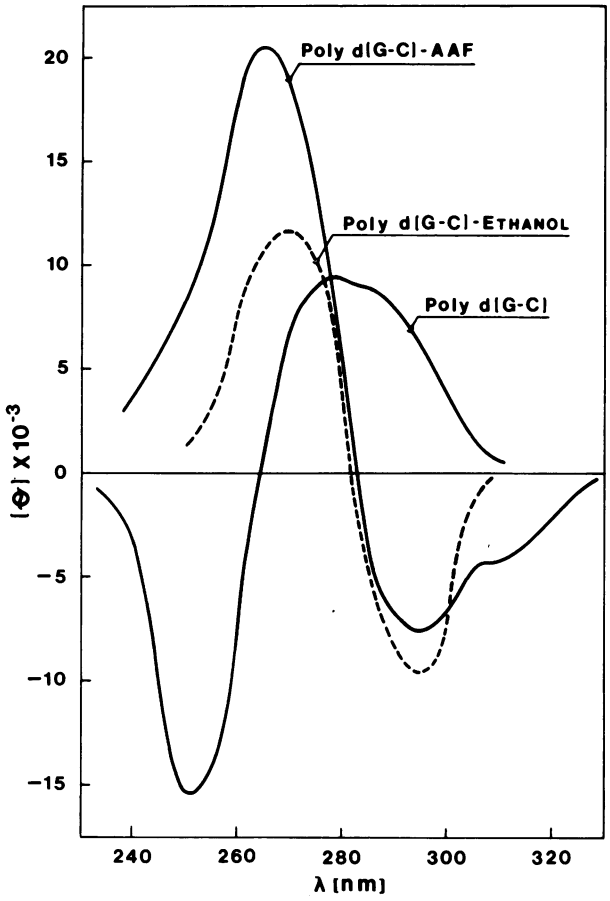

FIGURE 4. CD spectra of poly(dG-dC) · poly(dG-dC). In 1 *mM* phosphate buffer, in 60% ethanol, and modified by AAF to an extent of 28% in 1*mM* phosphate buffer. From Santella et al. (*13*).

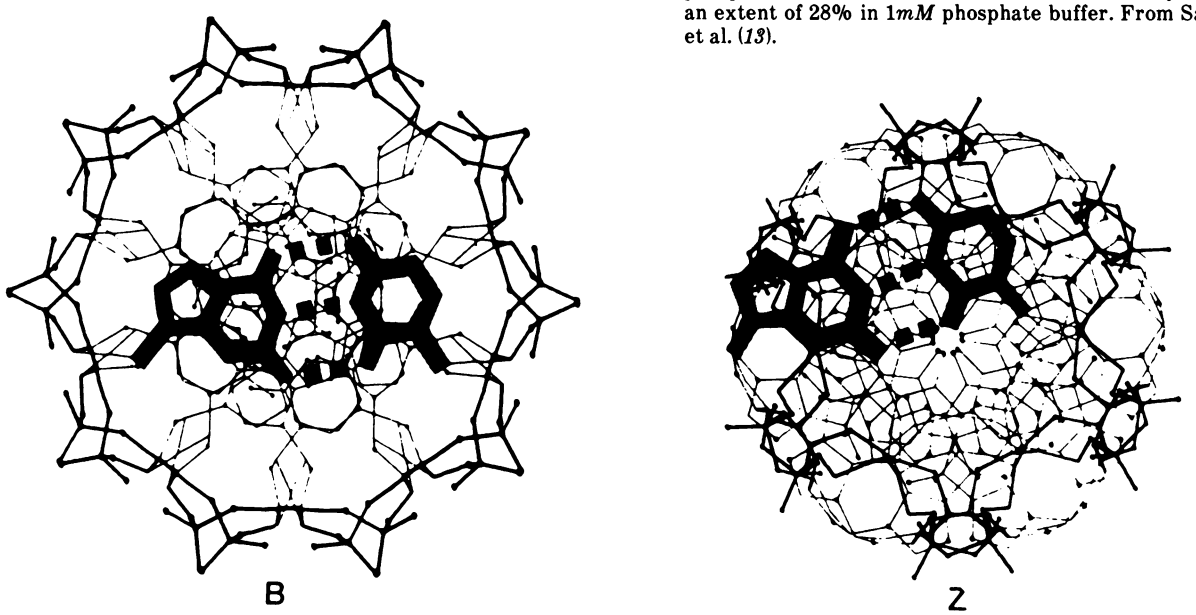

FIGURE 3. End views of the regular idealized helical forms of Z and B-DNA. Heavier lines are used for the phosphate-ribose backbone. A guanine-cytosine base pair is shown by shading. The difference in the positions of the base pairs is quite striking; they are near the center of B-DNA but at the periphery of Z-DNA. From Wang et al. (*6*) with permission.

trum resembles that of Z-DNA. With lower levels of modification (3%), the sample still shows a CD characteristic of B-DNA, but is converted to that of Z-DNA at lower ethanol concentrations than for unmodified poly(dG-dC)·poly(dG-dC) (Fig. 5). Sage and Leng (*14*) have also shown that with low levels of AAF modification, the polymer undergoes the B to Z transition at lower ethanol concentrations. On the other hand, studies on poly(dG)·poly(dC), a homopolymer which cannot undergo the B to Z transition (*8*), did not show any changes in CD spectra with high levels of AAF modification (*13*).

To obtain additional information about the conformation of the modified polymer, its susceptibility to S_1 nuclease digestion was determined. Table 1 shows that AAF-modified DNA and AAF-modified poly(dG)·poly(dC) were digested and therefore contain significant single-stranded regions. In contrast, poly(dG-dC)·poly(dG-dC) modified heavily with AAF was essentially resistant to S_1 nuclease digestion and must be double-stranded, proving that AAF modification does not induce localized regions of denaturation in this alternating purine-pyrimidine polymer.

Table 1. Nuclease S_1 digestion

	Modification, %	Digestion, %
DNA	0	5
DNA-AAF	20	75
Poly(dG-dC)-AAF	28	11
Poly(dG) · poly(dC)-AAF	19	59

Denatured sites in a double-stranded polymer can also be detected by radioimmunoassay using anticytidine antibodies (*15*). These antibodies react specifically with cytidine residues which are accessible in the single-stranded regions of a polymer. They precipitate a tracer of [^{3}H] denatured DNA (dDNA), but not native DNA (*16*). Addition of a competitor for reaction with the antibodies, such as nonlabeled dDNA, inhibits the precipitation of the radioactive tracer. This is shown in Figure 6, where addition of dDNA or DNA-AAF inhibits the precipitation of

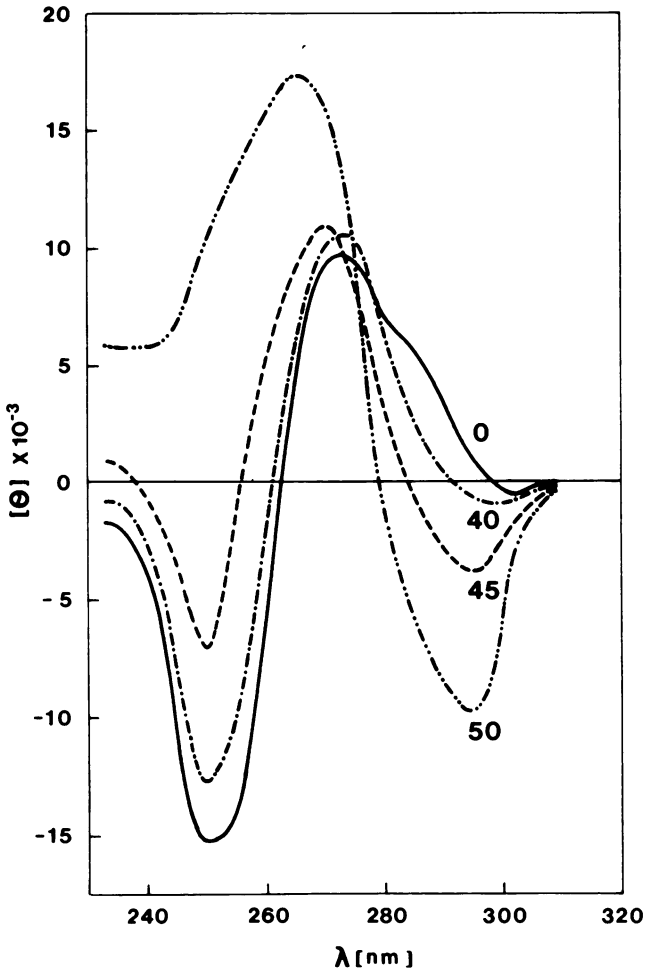

FIGURE 5. CD spectra of poly(dG-dC) · poly(dG-dC) modified with AAF to an extent of 3% at various ethanol concentrations: (——) in 1 *mM* phosphate buffer; (· ·) in 40% ethanol; (--) in 45% ethanol; (- · -) in 50% ethanol. From Santella et al. (*13*).

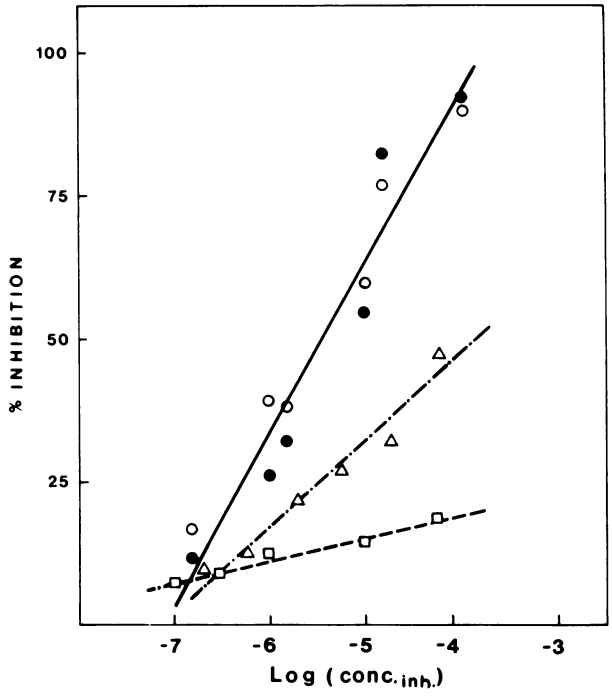

FIGURE 6. Radioimmunoassay at nonequilibrium conditions in which the binding of purified anti-C antibodies to [^{3}H]dDNA was measured in the presence of various concentrations of (●) dDNA; (○) DNA-AAF, 11% modified; (□) poly(dG-dC) · poly(dG-dC)-AAF, 21% modified; and (△) poly(dG) · poly(dC)-AAF, 5% modified. From Santella et al. (*15*).

the [^{3}H]dDNA by competing for reaction with the antibodies. Poly(dG-dC)·poly(dG-dC) with a 21% modification level does not inhibit the binding of the antibodies to the tracer, which indicates that this polymer does not react with the antibodies and therefore has no significant single-strand regions. In contrast, a sample of AAF-modified poly(dG)·(dC) does react with the antibodies and thus contains some denatured regions.

Potential Energy Calculations

In order to propose a model for the modified polymer, potential energy calculations were performed on the dCpdG model system in collaboration with Suse Broyde and Brian Hingerty (*15*). A low-energy conformation whose DNA backbone is very similar to that of the dCpdG segment of Z_1-DNA (*6*) is shown in Figure 7. The guanine is *syn* and approximately coplanar with cytidine, and the AAF residue is twisted nearly perpendicular to the G; the deoxyriboses are alternately C3′ *endo* in guanosine and C2′ *endo* in cytidine. This energy-minimized structure readily fits into the Z-DNA helix, as shown in Figure 8, where the computed adduct of Figure 7 is inserted into a model of the Z_1-DNA tetrameric duplex, dGpdCpdGpdC. The long axis of fluorene makes an angle of approximately 35° with the helix axis.

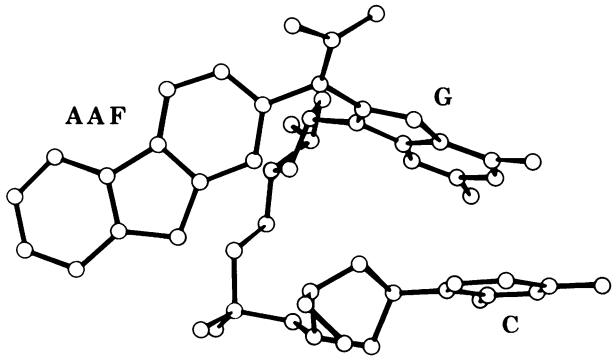

FIGURE 7. Minimum energy conformation of dCpdG-AAF with DNA backbone torsion angles similar to dCpdG segment of Z-DNA (*6*). From Santella et al. (*15*).

Taken together, the data with S_1 nuclease diges-

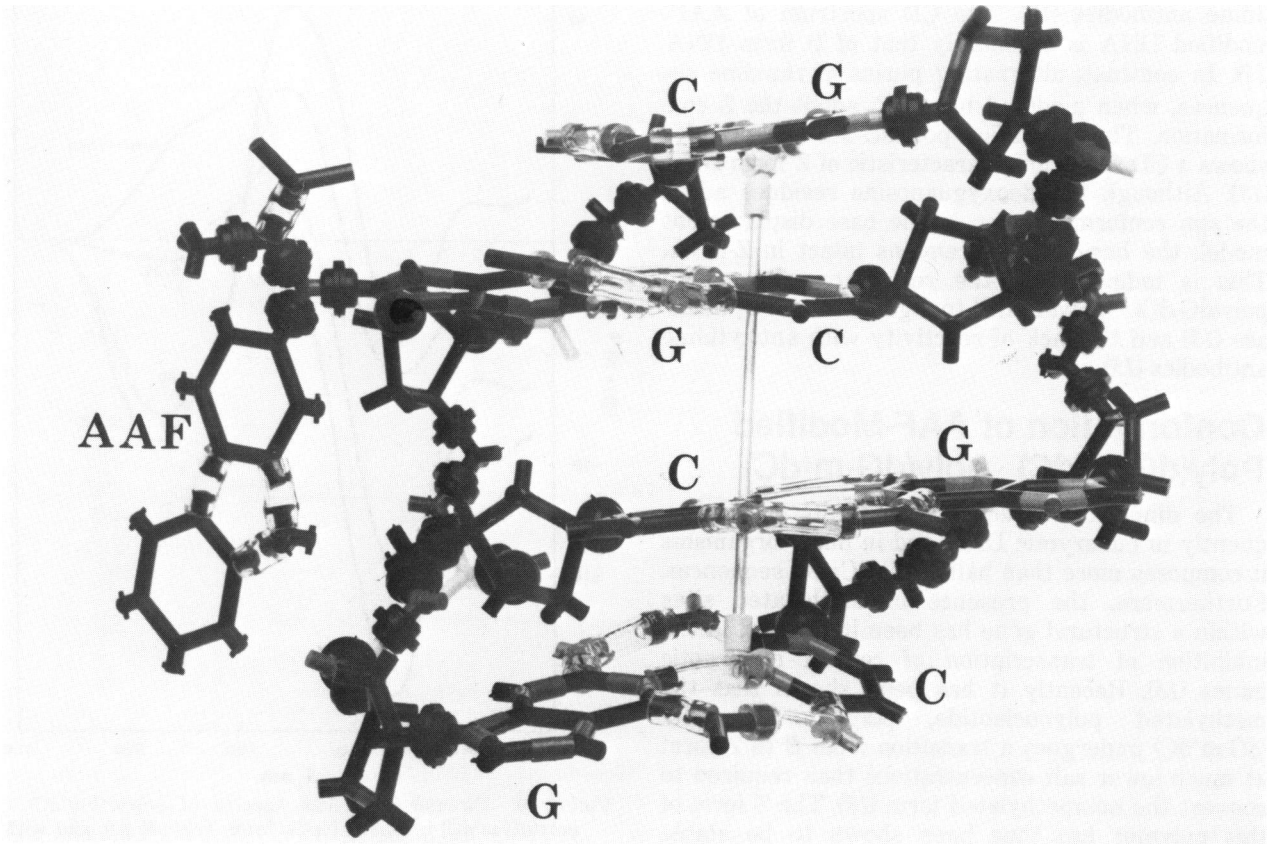

FIGURE 8. Minimum energy conformation of dCpdG-AAF shown in Figure 7 inserted into a Z_1-DNA tetrameric duplex of dGpdCpdGpdC. The AAF is linked externally to the Z-DNA helix and is mobile, with rotational flexibility about its long axis. From Santella et al. (*15*).

Table 2. Comparison of the properties of AAF-modified DNA and poly(dG-dC)·3poly(dGdC).

Properties	Type of AAF-modified polymers: DNA	[Poly (dG-dC)·poly(dG-dC)]
Conformation	Base displacement	Z-DNA
CD spectra	B-DNA type	Inverted B-DNA
Base pairing	Disrupted	Intact
Nuclease S_1 susceptibility	Sensitive	Resistant
Anticytidine antibodies	Reactive	Nonreactive

tion and anti-cytidine antibodies seem to be in accord with the suggestion that the inverted CD spectra are indicative of a Z-DNA type conformation Table 2 summarizes the data and indicates two different conformations for AAF-modified deoxyguanosine, base displacement, and Z-DNA, depending on whether the modified residues are in random or alternating purine-pyrimidine sequences. In random sequence DNA, the conformation with AAF modification is best represented by the base displacement model which involves disruption of base pairing and intercalation of the AAF residue. This partial denaturation of the DNA is detected by heat denaturation (*17*), increased susceptibility to digestion by S_1 nuclease (*3*, *4*), and increased reactivity with anticytidine antibodies (*15*). The CD spectrum of AAF-modified DNA is essentially that of B form DNA (*17*). In contrast, alternating purine-pyrimidine sequences, when modified by AAF, adopt the Z conformation. Thus, modified poly(dG-dC) · poly(dG-dC) shows a CD spectrum characteristic of Z form DNA (*13*). Although the deoxyguanosine residues adopt the *syn* conformation, as in the base displacement model, the base pairing remains intact in Z-DNA. This is indicated by the resistance of modified poly(dG-dC) · (poly(dG-dC) to digestion with S_1 nuclease (*13*) and the lack of reactivity with anticytidine antibodies (*15*).

Conformation of AAF-Modified Poly(dG-m^{5}dC) · poly(dG-m^{5}dC)

The dinucleotide sequence m^{5}dC-dG occurs frequently in eukaryotic DNA and in many organisms it composes more than half of all dCpdG sequences. Furthermore, the presence of methylated sites within a structural gene has been implicated in the inhibition of transcription of certain eukaryotic genes (*18*). Recently it has been shown that the methylated polynucleotide, poly(dG-m^{5}dC) · poly (dG-m^{5}dC) undergoes a transition from B to Z form at much lower salt concentrations than required to convert the nonmethylated form (*19*). The Z form of this polymer has thus been shown to be stable under typical physiological conditions.

We have investigated the conformational changes of poly(dG-m^{5}dC) · poly(dG-m^{5}dC) with AAF modification. The CD spectra of the polymer bound with various levels of AAF is shown in Figure 9. At modification levels above 6%, there begins to appear a negative band at 295 nm characteristic of Z-DNA. With 10.4% modification the polymer is completely in the Z form. Poly(dG-m^{5}dC) · poly(dG-m^{5}dC) undergoes a B to Z transition with increasing concentrations of Mg^{2+}, with a midpoint at 0.6 *mM* Mg^{2+} (*19*), compared to 0.7 *M* for the nonmethylated polymer. With low levels of AAF modification, it is possible to decrease further the amount of Mg^{2+} needed to induce the B to Z transition. A sample of poly(dG-

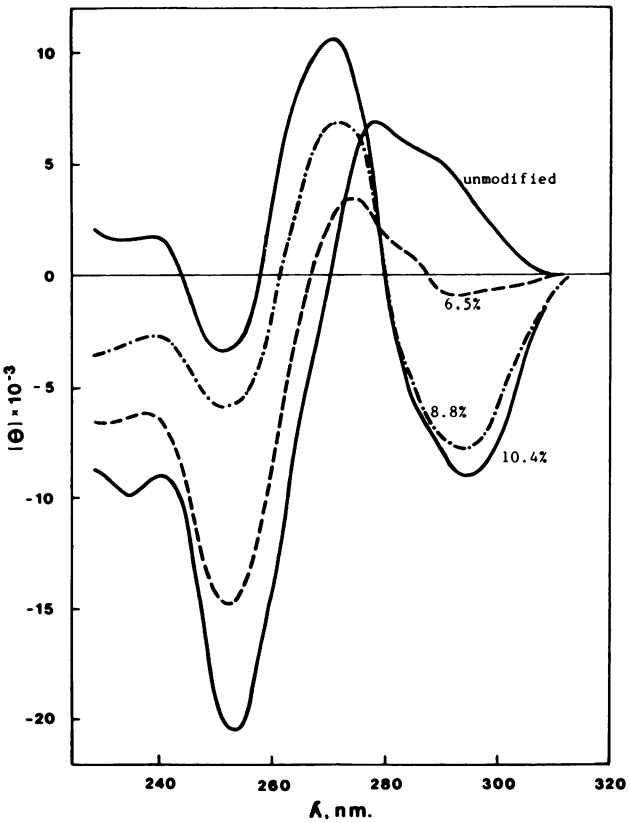

FIGURE 9. Circular dichroism spectra of poly(dG-m^{5}dC) poly(dG-m^{5}dC) in 50*mM* NaCl, 5*mM* Tris pH 8.0, and with various levels of AAF modification (——) control. (- -) 6.5% modification. (- · -) 8.8% modification, (· ·) 10.4% modification.

m⁵dC) · poly(dG-m⁵dC) with 3.5% AAF modification has a midpoint of transition at 0.3 *mM* Mg^{2-} (Fig. 10). Thus both methylation at the C5 of cytosine and AAF modification at C8 of guanosine favor induction of the Z conformation.

The susceptibility of this modified polymer to S_1 nuclease was also investigated. Figure 11 shows the level of digestion of the various polymers with time. Heat-denatured DNA is completely hydrolyzed by S_1 nuclease while native DNA is quite resistant. A DNA sample modified to a level of 8.5% showed about 50% digestion after 3 hr. In contrast, a sample of poly(dG-m⁵dC) · poly(dG-m⁵dC) with 17.5% modification shows only 14% digestion after 3 hr. incubation with S_1 nuclease. These results are similar to the resistance of modified poly(dG-dC) · poly(dG-dC) to S_1 nuclease digestion, and indicate that base pairing also remains intact when the methylated polymer is modified.

As a direct consequence of the *syn* conformation of the deoxyguanosine residues in Z-DNA, the C^8 position is exposed to the outer surface of the DNA molecule. The reactivity of these positions may, therefore, be increased. To investigate this possibility, the reactivity of poly(dG-dC) · poly(dG-dC) under

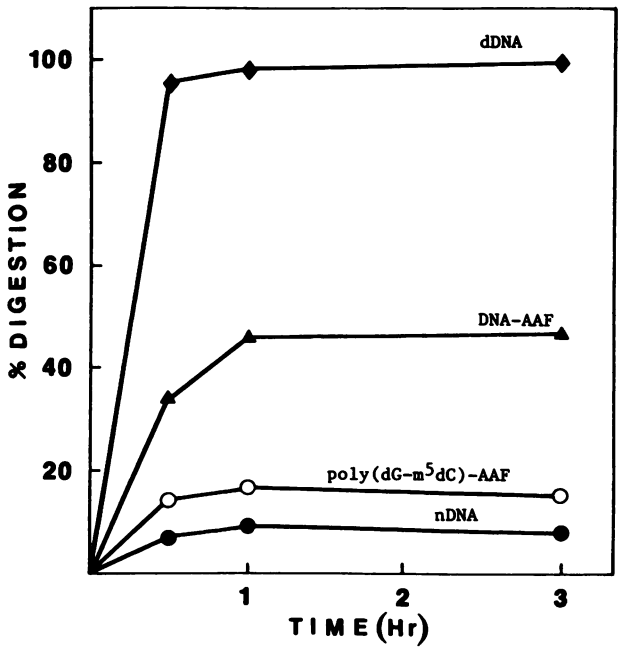

FIGURE 11. Nuclease S_1 digestion of (●) native calf thymus DNA, (◆) denatured calf thymus DNA, (○) poly(dG-m⁵dC) · poly(dG-m⁵dC) modified with AAF to an extent of 17.5%, and (▲) calf thymus DNA modified with AAF to an extent of 8.5%.

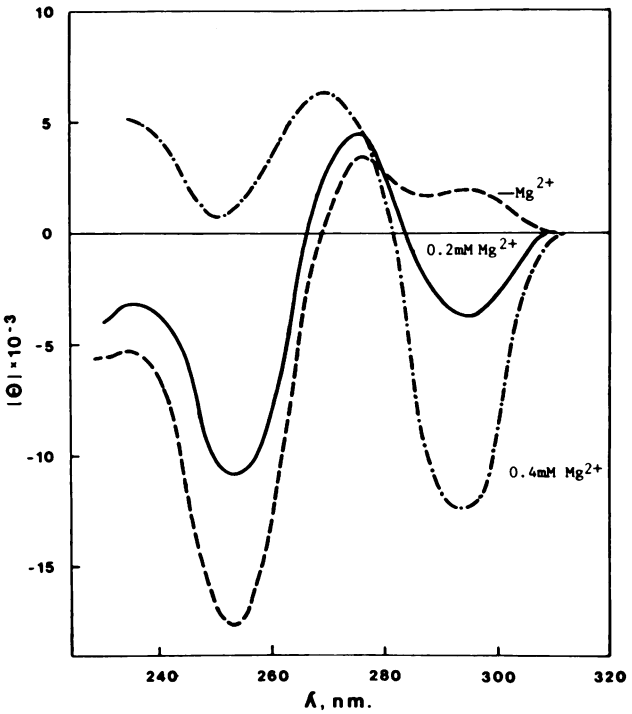

FIGURE 10. Circular dichroism spectra of poly(dG-m⁵dC) poly(dG-m⁵dC) modified by AAF to an extent of 3.5% in various concentrations of Mg^{2+}; (- -) No MG^{2+}; (——) 0.2 *mM* Mg^{2+}; (- · -) 0.4 *mM* Mg^{2+}. All samples contained 50 *mM* NaCl, 5*mM* Tris, pH 8.0.

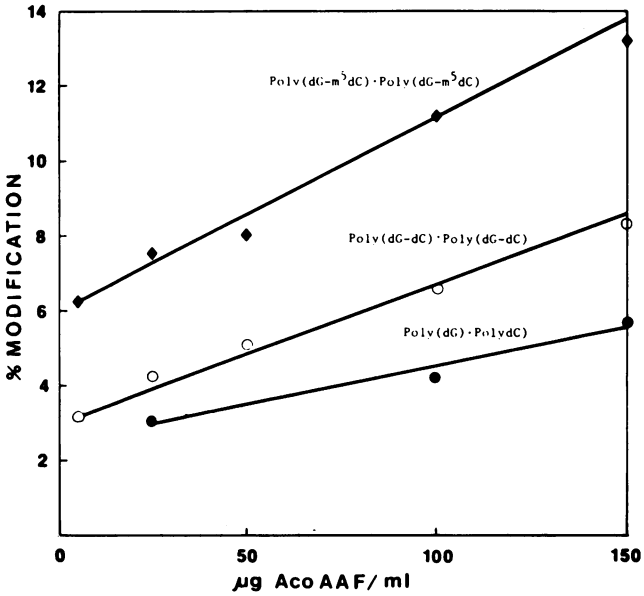

FIGURE 12. Extent of binding of AAF to synthetic polydeoxyribonucleotides in 50 *mM* NaCl, 5 *mM* Tris, pH 8.0: (●) poly(dG · poly(dC); (○) poly(dG-dC) · poly(dG-dC); (◆) poly(dG-m⁵dC) · poly(dG-m⁵dC).

various salt and ethanol concentrations, which stabilize the B or Z conformation, was studied. Unfortunately, those conditions which induced the Z conformation, even 40 μM cobalt hexamine chloride, inhibited the reaction of N-AcO-AAF with the polymer. Instead of kinetic binding experiments, the equilibrium binding level of the various polymers with increased levels of N-AcO-AAF was determined. The results shown in Figure 12 indicate that the order of reactivity follows the ease of formation of the Z conformation: poly(dG-m^{5}dC) · poly (dG-m^{5}dC) > poly(dG-dC) · poly(dG-dC) > poly(dG) · poly(dC). The higher reactivity of the alternating copolymer than the homopolymer had been seen previously (*20*). These results seem to indicate some preferential modification of the Z form, but further studies are necessary to confirm this result.

Discussion

Of critical importance in determining the role of Z-DNA *in vivo* is the prior demonstration of its presence in naturally occurring DNAs. While long tracts of alternating dG-dC sequences may be found, the best data, at present, is for seven base-pair lengths in the hairpin region of four related parvoviruses genomes (*21*). Recent studies have shown that stretches of up to 40 base pairs of dG-dC can be inserted into recombinant plasmids without interfering with their natural replication (*22*). These recombinants have also been shown to have both right- and left-handed conformations in the same molecule and indicates the possible role of DNA conformation in regulatory processes.

Indirect evidence for the existence of Z-DNA *in vivo* comes from studies using antibodies to Z-DNA (*23, 24*). These antibodies were shown to bind in reproducible patterns exclusively in interband regions of *Drosophila melanogaster* polytene chromosomes (*25*) which are associated with transcription of certain genes. Antibodies that react with Z-DNA were also found in the sera of mice with an autoimmune disease similar to human systemic lupus erythematosus. They occur spontaneously, but the immunogen is unknown.

A precondition for Z-DNA to be biologically active is its stabilization under physiological conditions as a left-handed segment in a mainly right-handed helix. One of the ways to achieve stabilization of Z-DNA *in vivo* is modification of DNA by C5 methylation of cytosine residues. Since methylation of the CpG sequence has been implicated in the inhibition of transcription of some eukaryotic genes, it is possible to assume that methylation induces the formation of Z-DNA segments which then can act as a conformational switch in the regulation of gene expression. Because AAF can also stabilize the Z-DNA conformation under physiological conditions, the proposal could be extended to chemical carcinogens such as AAF. This carcinogen could inhibit gene expression indirectly by causing a conformational switch in a region which, under normal conditions, would be transcribed. The feasibility of such a proposal is under our investigation.

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